Heat-shock Proteins of *Drosophila* Are Associated with Nuclease-resistant, High-salt-resistant Nuclear Structures

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ABSTRACT Proteins produced in cultured *Drosophila* cells during the heat-shock response (HSPs) were recently shown by autoradiography to be confined in large measure to the cell nucleus. We report here that nuclear HSPs are not associated with nucleosomes solubilized by treatment with staphylococcal nuclease at low ionic strength nor are HSPs released by extraction with high salt, which solubilizes most of the remaining histones and DNA. Possible functions of nuclear HSPs are discussed.

When *Drosophila* cells are subjected to a 10°C increase in temperature, synthesis of most normal RNAs and proteins is strongly repressed. Concurrently, one observes vigorous production of a few new (heat-shock) mRNAs and proteins (HSPs) (1–11). Many of the HSP genes have been cloned and mapped (12), and the chromatin structure of some of these loci has been analyzed (13–16). A considerable proportion of the newly synthesized HSPs were recently shown by autoradiography to be transported to the interior of the cell nucleus (1).

We report here the results of a biochemical study which confirms and extends the autoradiographic data (1). From 30 to 35% of the HSPs synthesized during a 90-min pulse of [^{35}S]methionine are found in the purified, extensively washed nuclei. Treatment of the nuclei with staphylococcal nuclease, while solubilizing up to 40% of the chromatin in the form of mono- and oligonucleosomes, leaves most of the HSPs within the insoluble pellet. After further extraction with 2 M NaCl, which removes most of the remaining DNA and histones, HSPs continue to sediment with the nuclease-resistant, high salt-resistant insoluble fraction.

Because transcription is one of the processes most strongly affected by the heat-shock response, it seems likely that nuclear HSPs play a role in protecting the template-active portion of the genome from adverse effects of heat shock, as suggested by Velazquez et al. (1). On the basis of our results, it appears that such protection arises from structures which are physically distinct from solubilizable chromatin (nucleosomes).

MATERIALS AND METHODS

Drosophila melanogaster cells (Schneider line 2; gift of Dr. M. L. Pardue) were grown in spinner culture at 25°C as described by Lengyel et al. (17). Cells were pelleted and resuspended in methionine-free medium, allowed to recover for 15 min at 25°C, and then shifted to 35°C for 30 min. Thereafter, [^{35}S]methionine (400 Ci/mmol; New England Nuclear, Boston, Mass.) was added to 50 μCi/ml and incubation at 35°C was continued for an additional 90 min. Cells (from 5–10 × 10^8 per preparation) were then pelleted at 4°C, washed once with 0.14 M NaCl, 5 mM Tris-HCl, pH 7.5, and lysed by gentle pipetting in 0.25% Nonidet P-40, 0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-HCl, pH 7.5. An aliquot of the lysed cell suspension was saved. Nuclei were pelleted at 2,500 *g* for 5 min, and the supernate, containing cytosol, was saved. Nuclei were washed three times in the lysis buffer and three times with digestion buffer (0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM PMSF, 10 mM Tris-HCl, pH 7.5). After resuspension in 1.5 ml of digestion buffer to a final DNA concentration of ~1 mg/ml, the nuclei were digested at 37°C with staphylococcal nuclease at 5 μg/ml for various time intervals. Samples (0.75 ml) were then cooled to 4°C, centrifuged at 2,500 *g* for 10 min, and the supernates (designated below as first supernates) were saved. Pellets were resuspended in 0.75 ml of 0.5 mM Na-EGTA, 1 mM Na-EDTA, 1 mM Tris-HCl, pH 7.5, and centrifuged at 12,500 *g* for 5 min. Second supernates were saved and the swollen pellets were resuspended in the same buffer.

The pellets were further fractionated by extraction with 2 M NaCl. 4 M NaCl and 0.1 M Tris-HCl, pH 7.5 were added to final concentrations of 2 M and 10 mM, respectively. After 20 min at 4°C, the samples were centrifuged at 12,500 *g* for 10 min. The clear supernates were removed from the compact, granular pellets. The supernates were made 25% in CCl_3COOH, held at 4°C for 1 h, and centrifuged at 12,500 *g* for 10 min. The precipitated protein was washed with acetone–0.1 N HCl, then with acetone, dried under vacuum, and redissolved in an SDS-containing sample buffer.

Analysis of proteins in the fractions obtained was carried out using the SDS PAGE system of Laemmli (18) as described by Thomas and Kornberg (19). The gels (1.5-mm thick, 30-cm long) were stained with Coomassie Blue to visualize total protein patterns and thereafter processed for fluorography as described by Laskey and Mills (20).

Control cells were grown and labeled with [^{35}S]methionine at 25°C, spun, resuspended, fractionated, and analyzed exactly as were the heat shock-cells, except for the temperature shift.

RESULTS AND DISCUSSION

Fig. 1, lane *A1* displays an SDS electrophoretic pattern of [^{35}S]proteins from whole, heat-shocked *Drosophila* cells. Several strongly labeled bands correspond to the previously characterized proteins induced by heat shock (2, 11). Most of the [^{35}S]methionine label has been incorporated into the heat-shock proteins, with the remainder being incorporated into the histones (see Fig. 3 for their locations on the electrophoretogram) and into a heterogeneous spectrum of other proteins (Fig. 1, lane *A1*). No HSP bands are seen in the whole cell [^{35}S]protein pattern of control, nonshocked cells, with the possible exception

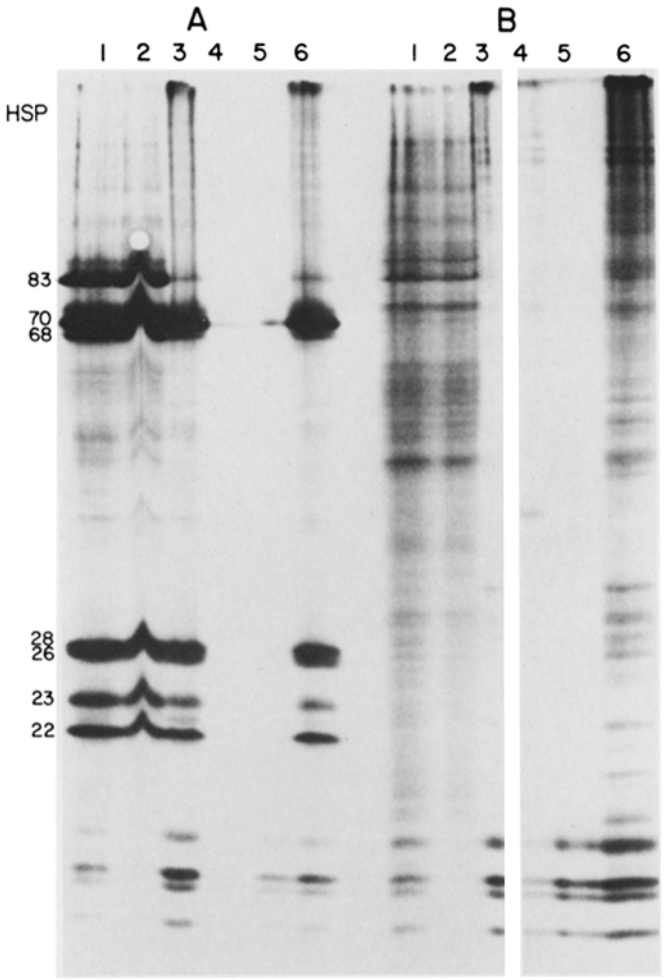

FIGURE 1 Distribution of heat-shock proteins among subcellular fractions. Panel *A*, patterns of [^{35}S]proteins from heat-shocked cells: *A1*, total cell lysate; *A2*, an equal volume of cytosol-containing supernate; *A3*, washed nuclei (from a threefold higher number of cells relative to that in *A1* and *A2*); *A4*, first supernate from nuclease-treated nuclei (sample volume equivalent to that in *A3*); *A5*, an equal volume of the second supernate (~25% of the chromatin was solubilized and recovered in the second supernate of this particular nuclease digest); and *A6*, pellet left after taking first and second supernates (sample volume equivalent to sample volumes in *A3–A5*). Designations to the left of lane *A1* indicate apparent molecular weights of HSP's in kilodaltons. Panel *B*, patterns of [^{35}S]proteins from control (nonheat-shocked) cells: lanes *B1–B6* are homologous to lanes *A1–A6*, respectively. Fluorographic exposure was for 4 h in *A1–A6*, and *B1–B3*, and for 16 h in *B4–B6*.

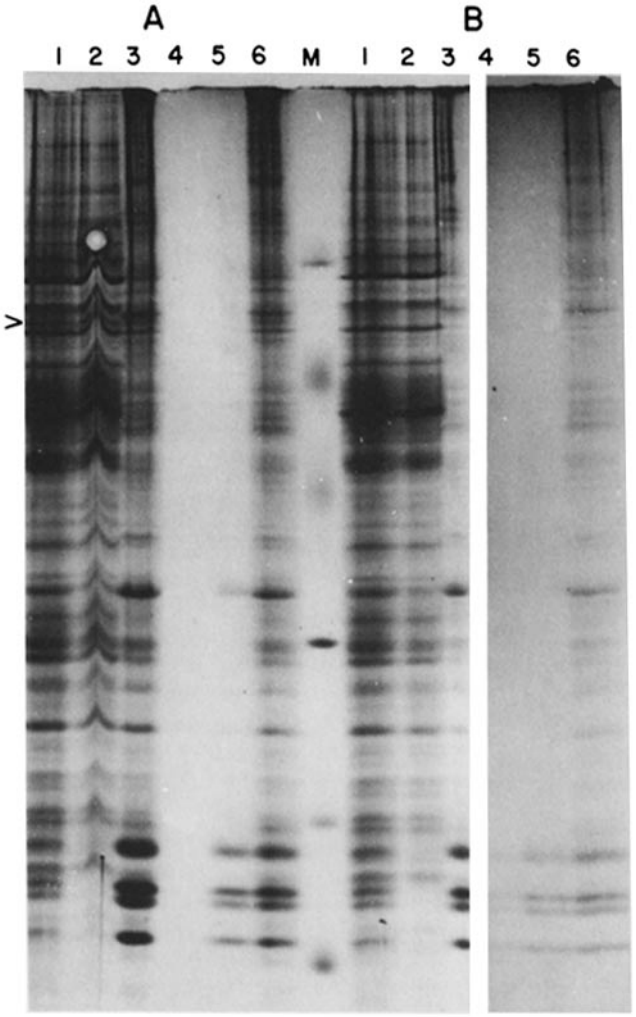

FIGURE 2 Coomassie Blue–stained protein patterns. *A1–A6* and *B1–B6* are identical to Fig. 1, *A1–A6* and *B1–B6*, respectively. *M*, marker proteins: phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), α-lactalbumin (20,000), and soybean trypsin inhibitor (14,000) (Pharmacia). The arrowhead next to lane *A1* identifies HSP-70, absent from control cells (*B1*).

of HSP-83 (Fig. 1, lane *B1*). Coomassie Blue–stained protein patterns of whole cell lysates from shocked and unshocked cells (Fig. 2, lanes *A1* and *B1*) show that under conditions of heat shock used in this work the relative mass of the HSPs in heat-shocked cells is still low but already detectable (arrow in Fig. 2, lane *A1*). Fig. 1, lanes *A2* and *A3*, shows that although a considerable proportion of the HSPs are recovered in the cytosol-containing fraction (lane *A2*), extensively washed nuclei also contain HSPs (Fig. 1, lane *A3*; cf. lane *A2*).

Careful comparisons (made using a densitometer) of relative intensities of the HSP bands in the various fractions at different levels of fluorographic exposure show that from 30 to 35% of each HSP is located in the extensively washed nuclear fraction, with the exception of HSP-83, which is almost entirely cytoplasmic (Fig. 1, lane *A2*; cf. lane *A3*). It is not known whether the absence of HSP-83 from the nuclear fraction is due to its cytoplasmic localization in vivo or to preferential loss during isolation of the nuclei. Note also that HSP-23 is slightly underrepresented in the nucleus as compared with the other HSPs (Fig. 1, lanes *A2* and *A3*). It should be stressed that heat-shock nuclei were pelleted and resuspended exactly like nuclei from nonshocked cells; they were in no sense "sticky." Furthermore, the Coomassie Blue–stained pattern of proteins from heat-shocked nuclei was practically indistinguishable from the control pattern (Fig. 2, lanes *A3*, *B3*, and data not shown), indicating that little if any cytoplasmic contamination of isolated nuclei results from heat shock.

These data on the nuclear localization of a substantial proportion of *Drosophila* HSPs are in accord with both autoradiographic data of Velazquez et al. (1) and a recent report that a specific 34,000-dalton HSP of *Chironomus tentans* was greatly enriched in nuclear preparations from microdissected *Chironomus* nuclei (21). A similar result was recently reported for *Tetrahymena* (22). It should be emphasized that autoradiographic data of the type produced by Velazquez et al. (1) are

not subject to the same sort of artifacts possible for biochemical fractionations and vice-versa. Therefore, a complete agreement between the autoradiographic observation of an intranuclear localization of a considerable proportion of HSPs in cultured *Drosophila* cells (1) and the biochemical data (Fig. 1) strongly suggests that the HSPs in the purified nuclear preparations are indeed intranuclear proteins.

The next question is whether or not nuclear HSPs are associated with histones in the nucleosomes of chromatin fibers. Very little chromatin is released in the first supernate (in the presence of Mg^{2+}) after staphylococcal nuclease digestion of *Drosophila* nuclei from heat-shocked cells (for details of the preparation, see Materials and Methods), as shown by the virtual absence of core histone bands in Fig. 1, lane *A4*. Only trace amounts of the HSPs are found in the first supernate (Fig. 1, lane *A4*; cf. lane *A6*). The second, low ionic strength supernates contain 20–40% of the total chromatin in these digests, based on relative fluorographic and staining intensities of histone bands (lanes *A5* and *A6* in Fig. 1 and Fig. 2) and on direct measurement of the amount of released DNA (data not shown). Variations in the yield of soluble chromatin, ~25% for the heat-shocked sample (Fig. 1, lane *A5*) and ~35% for the nonshocked sample (Fig. 1, lane *B5*), are due primarily to different extents of staphylococcal nuclease digestion. Longer fluorographic exposures of lanes *A5* and *B5* in Fig. 1 confirm that, in addition to the core histones, both histone H1 and a heterogeneous set of nonhistone proteins are present in the nuclease-solubilized chromatin (data not shown). The histone H1 band can be seen in the corresponding Coomassie Blue-stained pattern (Fig. 2, lane *A5*) between ovalbumin (43,000 dalton) and carbonic anhydrase (30,000 dalton) molecular weight markers (Fig. 2, lane *M*).

Little if any HSP is released with the solubilized chromatin present in the second supernate in the form of oligo- and mononucleosomes (Fig. 1, lane *A5*; cf. lane *A6*) (23, 24). Staphylococcal nuclease digestion of nuclei is also reported to release a considerable proportion of nuclear ribonucleoproteins (RNPs), mainly in the form of small RNP particles and oligonucleotides (24). In contrast, >95% of the nuclear HSPs are not released after staphylococcal nuclease digestion of the nuclei, which solubilizes a significant proportion of chromatin (nucleosomes) (Fig. 1, lanes *A4–A6*). We conclude that the nuclear HSPs are not associated directly with the nucleosomes released by nuclease at low ionic strength. While this manuscript was being written, Guttman et al. (22) reported a similar result with nuclear HSP's of *Tetrahymena*.

Strikingly, the nuclear HSPs are not released even after treatment of the insoluble nuclear fraction with 2 M NaCl, which solubilizes most of the remaining histones and DNA (Fig. 3, lanes A1–A3). Longer fluorographic exposures of Fig. 3 confirm that virtually all histones are extracted with 2 M NaCl (Fig. 3, lane *A2* and data not shown), while >95% of the HSPs remain with the 2 M NaCl-insoluble pellet (Fig. 3, lane *A3*). Subjecting cytoplasmic HSPs (Fig. 1, lane *A2*) to the treatment with 2 M NaCl does not result in any HSP precipitation (data not shown), indicating that at least cytoplasmic HSPs themselves are soluble in 2 M NaCl.

The high salt-resistant fraction, in addition to the peripherally located nuclear lamina (25), is believed to contain components of an intranuclear fibrous scaffold or matrix (26–34). The precise nature of interactions of nuclear HSPs with elements of the high salt-resistant fraction, operationally defined as a nuclear scaffold, remains unknown and, in fact, a *Dro-*

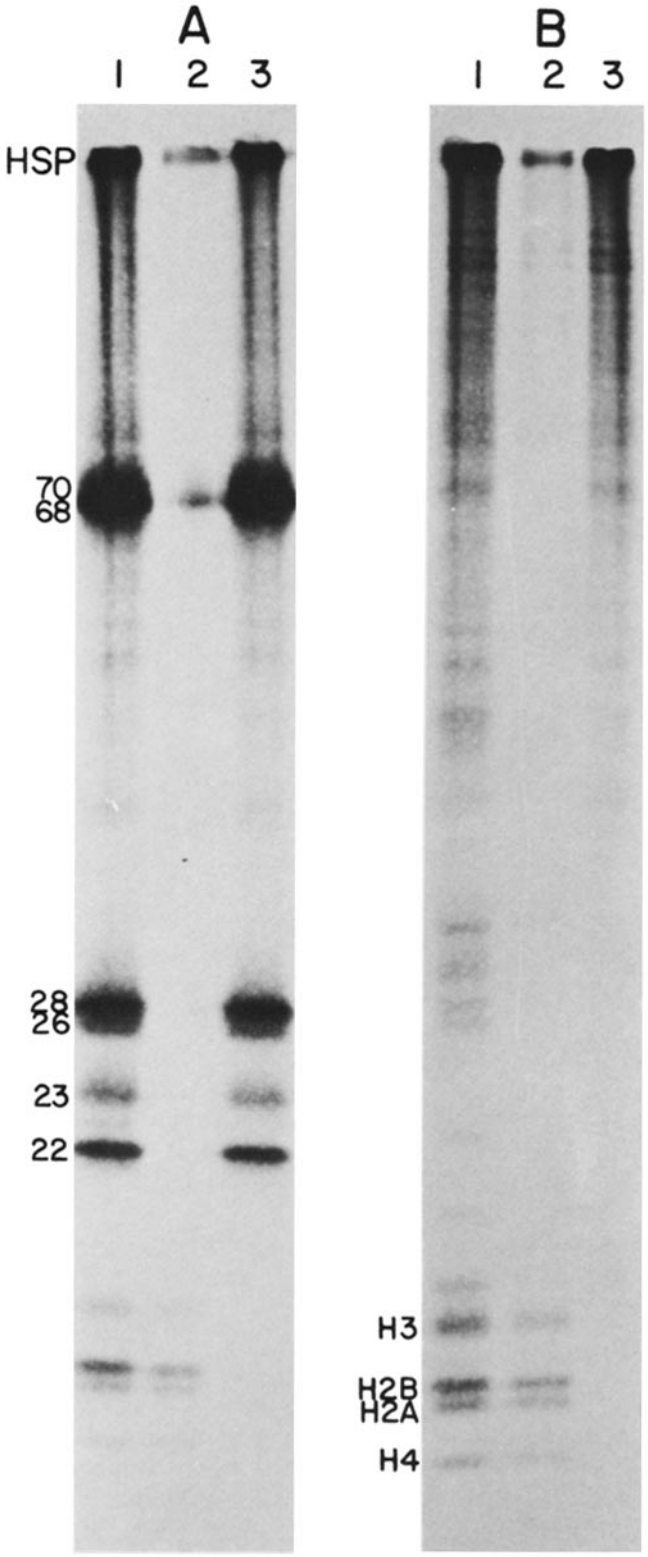

FIGURE 3 Association of heat-shock proteins with high salt-resistant nuclear structures. Panel *A*: patterns of [^{35}S]proteins from heat-shocked cells: *A1*, insoluble pellet after staphylococcal nuclease digestion of nuclei from heat-shocked cells (same sample as lane *A6* in Fig. 1); *A2*, 2 M NaCl extract of the sample shown in *A1*; *A3*, 2 M NaCl-insoluble material from the sample shown in *A1*. Panel *B*: patterns of [^{35}S]proteins from control, nonheat-shocked cells. Lanes *B1–B3* are homologous to lanes *A1–A3*, respectively. Lane *B1* is the same sample as lane *B6* in Fig. 1 *B*. Designations next to lane *B1* indicate *Drosophila* histone fractions (38).

sophila nuclear scaffold has not yet been rigorously characterized by anyone. The relatively well-defined HSP proteins can now be used as a probe to study poorly understood relationships between the lamina, nuclear scaffold, and chromatin. Velazquez et al. (1) found that HSP autoradiographic grains are distributed throughout the interphase, nonpolytene nucleus, absent only from the condensed heterochromatin. This suggests that nuclear HSPs are part of an intranuclear, not exclusively peripheral (lamina) structure.

The phenomenon of an induced synthesis of a set of specific proteins upon a heat or metabolic shock has recently been observed in different insect, mammalian, plant, fungal, and protozoan cells (1, 2, 21, 22, 35–37) and thus appears to be a universal one. On the basis of our work and that of others (1, 21, 22), we postulate a role of *Drosophila* HSPs in preservation of the spatial organization of transcriptionally active chromatin. Therefore, we predict that in all eukaryotic cells in which the heat-shock response is observed, at lease some HSPs will be identified as components of high salt-resistant nuclear structures. We further suggest that additional sites of anchorage between chromatin and HSP-containing nuclear structures may occur when HSPs accumulate in the nucleus during the heat-shock response.

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